JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2008, p. 3303–3310 Vol. 46, No. 10
0095-1137/08/$08.00+0 doi:10.1128/JCM.00644-08

Biological and Phylogenetic Characterization of Pigeon Paramyxovirus Serotype 1 Circulating in Wild North American Pigeons and Doves[▽]

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Received 4 April 2008/Returned for modification 18 June 2008/Accepted 7 August 2008

As part of West Nile virus surveillance programs in Rhode Island and eastern Texas between 2000 and 2007, brain tissue was collected from 5,608 dead birds representing 21 avian orders found in public places or reported by homeowners. Fifteen Newcastle disease virus isolates were recovered only from birds of the order Columbiformes and were positively identified by the USDA-validated real-time reverse transcription-PCR assay targeting the matrix gene and more specifically as pigeon paramyxovirus serotype 1 (PPMV-1) by hemagglutinin inhibition with monoclonal antibodies. Based upon partial genomic sequencing and phylogenetic analysis, the newly isolated viruses represent a distinct sublineage within class II genotype VIb. All of the viruses (15/15) were classified as virulent based upon their fusion cleavage site motif (^{112}RRKKRF^{117}) and intracerebral pathogenicity indices of >0.7 (ranging from 0.98 to1.35); however, these viruses escaped detection by the fusion gene-based real-time PCR test for virulence. Modifications introduced to the probe site of the fusion gene-based assay allowed rapid virulence detection within this distinct sublineage.

Virulent forms of Newcastle disease (ND) virus (NDV; also known as avian paramyxovirus serotype 1) are a major economic concern for poultry producers worldwide (6). While there have been previous outbreaks of disease due to virulent NDV infections, poultry in the United States is currently considered free of ND. Disease control programs to prevent the reintroduction of virulent NDV into domestic poultry flocks include vaccination and quarantine of imported birds and must be complemented with monitoring programs. Rapid diagnostic assays such as real-time reverse transcription (RT)-PCR aid in the timely detection of potential outbreaks and are a crucial part of these efforts (15, 40).

At least three major panzootics of ND have been reported in the past 80 years. The first was recognized during the mid 1920s and affected birds in Indonesia and England (5), the second was identified in Europe during the late 1960s but was thought to have originated in Asia (18), and a third panzootic involving a pigeon-adapted variant of avian paramyxovirus serotype 1 that likely originated in the Middle East was detected during the 1980s (19) and continues around the world (2, 5, 32). Pigeon paramyxovirus serotype 1 (PPMV-1) affects pigeons and doves (Columbiformes) and is known to infect poultry (9, 12, 23, 25, 39). Widespread in racing pigeon populations in many countries of the world, PPMV-1 spread to wild birds such as wood pigeons (*Columba palumbus*) and Eurasian collared doves (*Streptopelia decaocto*; ECD) (35, 38). In countries with large populations of Columbiformes, the disease is now considered to be endemic (2, 31). For example, in the United States the virus is believed to be endemic and outbreaks were reported in 1998 in Texas and Georgia (25); however, PPMV-1 strains from the United States have rarely been phylogenetically characterized. Although many countries maintain compulsory vaccination of racing pigeons, there is no form of disease control in wild pigeons, which frequently have contact with backyard and free-range poultry (8, 9, 36). The virulence of PPMV-1 has been reported to be variable (14, 22, 31), and at times the only clinical sign of PPMV-1 infection in layer chickens was a drop in egg production, misshaped eggs, and soft egg shells (7). However, increased pathogenicity in chickens has been identified when PPMV-1 is passaged in chickens or embryonated eggs, indicating that viruses currently circulating among pigeon populations could lead to ND outbreaks (7, 22, 24). A recent study demonstrated that 11 (78.5%) of 14 virulent poultry NDV isolates from China obtained between 1996 and 2005 were typical of PPMV-1 strains that clustered into a single genetic lineage, 4b (2), or genotype VIb (28).

Rapid diagnosis of PPMV-1 is achieved with the USDA-validated real-time RT-PCR assay targeting the matrix gene (M-gene assay) (15, 40). The M-gene assay detects most class II NDV strains, including members of the PPMV-1 subgroup. Another real-time assay is employed that allows discrimination between virulent and avirulent isolates and is directed at the fusion gene (F-gene assay) (40). Upon initial testing of the F-gene assay, one member of the PPMV-1 subgroup was found to escape detection (Dove/Italy/2736/2000 [DoveIT]) (20, 40). When the sequence of the DoveIT isolate was compared to the sequences of the fusion test primers and probe, several mismatches were identified in the primer sequences, as well as 4-nucleotide (nt) mismatches at the fusion test probe site (20). The genetic differences between the DoveIT isolate and the fusion test probe appeared to be responsible for the test failure. Data from this study also revealed that the DoveIT isolate

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▽ Published ahead of print on 20 August 2008.

TABLE 1. NDV fusion gene sequences[a] used in the analysis described in this report[b]

GenBank accession no.	Species	Tree name	Figure(s)
AY150141	Chicken	94DE001CKN	3
AY175776	Chicken	95TR002CKN	3
AY288995	Dove	00IT003DVE	2, 3
AF456439	Goose	98CN004GSE	2, 3
AJ880277	Pigeon	82IT005PGN	3
AY150115	Pigeon	87IT006PGN	3
AY471758	Pigeon	90UK007PGN	3
AY150132	Pigeon	92ES008PGN	3
AY150133	Pigeon	93DE009PGN	3
AY471761	Pigeon	93DK010PGN	3
AY150140	Pigeon	94DE011PGN	3
AY175753	Pigeon	94DE012PGN	3
AY471762	Pigeon	94DE013PGN	3
AY471767	Pigeon	94DE014PGN	3
AY471764	Pigeon	94UK015PGN	3
AY471759	Pigeon	95AT016PGN	3
AY471757	Pigeon	95DE017PGN	3
AY471772	Pigeon	95DE018PGN	3
AY471777	Pigeon	95DE019PGN	3
AB070419	Pigeon	95JP020PGN	3
AY471781	Pigeon	96AE021PGN	3
AY390291	Pigeon	96CN022PGN	3
DQ417113	Pigeon	96CN023PGN	2, 3
AY471768	Pigeon	96IE024PGN	3
AB070423	Pigeon	96JP025PGN	3
AY471782	Pigeon	97AE026PGN	3
AY734536	Pigeon	97AR027PGN	2, 3
AY471771	Pigeon	97AT028PGN	3
AY471778	Pigeon	97AT029PGN	3
AY390288	Pigeon	97CN030PGN	3
AB070426	Pigeon	97JP031PGN	3
AY471779	Pigeon	98AE032PGN	3
AY471760	Pigeon	98AT033PGN	3
AF358785	Pigeon	98CN034PGN	2, 3
AY150149	Pigeon	98DE035PGN	3
AY471765	Pigeon	98FR036PGN	3
AY175760	Pigeon	98SA037PGN	3
AY471766	Pigeon	98UK038PGN	3
AY444501	Pigeon	98US039PGN	3
AY471780	Pigeon	99AE040PGN	3
AY734535	Pigeon	99AR041PGN	2, 3
AY390290	Pigeon	99CN042PGN	3
AY150153	Pigeon	99DE043PGN	3
AY288996	Pigeon	00IT044PGN	3
AB070434	Pigeon	00JP045PGN	3
DQ439885	Pigeon	05CN046PGN	3
DQ904250	Pigeon	06CA047PGN	3
EU477193	ECD	03US434ECD	3
EU477194	ECD	04US435ECD	3
EU477195	ECD	06US436ECD	3
EU477196	ECD	07US437ECD	3
EU477197	ECD	07US438ECD	3
EU477188	Pigeon	04US440PGN	2, 3
EU477189	Pigeon	00US441PGN	2, 3
EU477198	Pigeon	04US443PGN	3
EU477190	Pigeon	04US444RDV	2, 3
EU477199	ECD	04US445ECD	3
EU477191	ECD	04US446ECD	2, 3
EU477200	Mourning dove	04US447MDV	3
EU477201	ECD	04US448ECD	3
EU477202	Pigeon	05US449RDV	3
EU477192	ECD	05US450ECD	2, 3
AY288995	Dove	00IT003DVE	2, 3
AF456439	Goose	98CN004GSE	2, 3
DQ417113	Pigeon	96CN023PGN	2, 3
AY734536	Pigeon	97AR027PGN	2, 3
AF358785	Pigeon	98CN034PGN	2, 3
AY734535	Pigeon	99AR041PGN	2, 3
AY562986	Anhinga	93US048ANH	2
M24695	Chicken	47US049B1	2
AY965077	Baikal teal	02RU050BKT	2
X04719	Beaudette C	45US051BEA	2
AY288997	Chicken	90KE052CKN	2
AY288999	Chicken	96MX053CKN	2
AY338284	Chicken	98CN054CKN	2
DQ067447	Chicken	00CN055CKN	2
AY288993	Chicken	00HN056CKN	2
AY288994	Chicken	00IT057CKN	2
AY427817	Chicken	02CN058CKN	2
DQ486859	Chicken	03CN059CKN	2
AY562985	Cockatoo	90ID061CCT	2
M24705	Duck	76JP062DCK	2
AY727881	Duck	98AR063DCK	2
DQ439947	Duck	01RU064DCK	2
AY626266	Duck	01US065DCK	2
AY562988	Fontana	72US066FON	2
AY028995	Fowl	96CN067FWL	2
AF458015	Fowl	97CN068FWL	2
AF364835	Fowl	98CN069FWL	2
AF458010	Fowl	00CN070FWL	2
AF358786	Fowl	00TW071FWL	2
AY562987	Game fowl	02US072GFL	2
EF540729	Goose	97CN073GSE	2
AF162714	Goose	97CN074GSE	2
AF456438	Goose	00CN075GSE	2
AF534997	Goose	00CN076GSE	2
AF456442	Goose	01CN077GSE	2
AF456443	Goose	01CN078GSE	2
AF473851	Goose	02CN079GSE	2
EF211814	Goose	06CN080GSE	2
EF579734	Goose	06CN081GSE	2
EF564813	Green-winged teal	98US082GWT	2
AY741404	Herts	33UK083HRT	2
AY845400	LaSota	46US084LAS	2
EF564832	Mallard	86US085MLD	2
EF565079	Mallard	99US086MLD	2
EF564829	Mallard	01US087MLD	2
EF564830	Mallard	01US088MLD	2
EF564824	Mallard	02US089MLD	2
EF564825	Mallard	02US090MLD	2
EF564828	Mallard	03US091MLD	2
EF564821	Mallard	04US092MLD	2
EF592500	Mallard	05CN093MLD	2
AY288987	Mixed	71US094MXD	2
EF564826	Northern pintail	89US095NOP	2
AY253912	Parrot	01CN096PAR	2
DQ080015	Penguin	99CN097PEN	2
AF109885	Pigeon	84UK098PGN	2
EF520716	Pigeon	84US099PGN	2
EF026579	Pigeon	98BE100PGN	2
AY928933	Poultry	75IR103PGN	2
AF217084	QV4	66AU104QV4	2
EF564819	Red knot	00US105RKN	2
EF564816	Red knot	01US106RKN	2
EF564817	Ruddy turnstone	02US107RDT	2
EF564831	Ruddy turnstone	04US108RDT	2
AY727882	Swan	00AR109SWN	2
AY289001	Turkey	92US111TKY	2
AY562991	Ulster	67UK112ULS	2
AF400615	Unknown	97CN113UNK	2
AY935489	Unknown	01AU114UNK	2
DQ227246	Unknown	02CN115UNK	2
DQ858357	Unknown	03CN116UNK	2
DQ227251	Unknown	03CN117UNK	2
DQ417111	Unknown	03CN118UNK	2
DQ858356	Unknown	06CN119UNK	2
EU477188	Dove	04US440DVE	2
EU477189	Pigeon	00US441PGN	2
EU477190	Pigeon	04US444RDV	2
EU477191	ECD	04US446ECD	2
EU477192	ECD	05US450ECD	2

[a] $n = 141$.

[b] Each virus designation represents a 10-character name containing the two-digit year of collection, the two-letter International Organization for Standardization (ISO) country code abbreviation, the three-digit unique virus identification number, and the three-letter species abbreviation. The sequences used for phylogenic analysis in Fig. 2 and 3 are indicated in the last column.

was phylogenetically related to a subset of PPMV-1 strains belonging to lineage 4bii (genotype VIb1) (2, 38). Eighty-six percent of these isolates (32/37) contained identical mismatches with DoveIT, and it was predicted that viruses from this sublineage were unlikely to be detected by the F-gene assay.

To improve our understanding of the distribution and evolution of PPMV-1, we examined representative strains of the

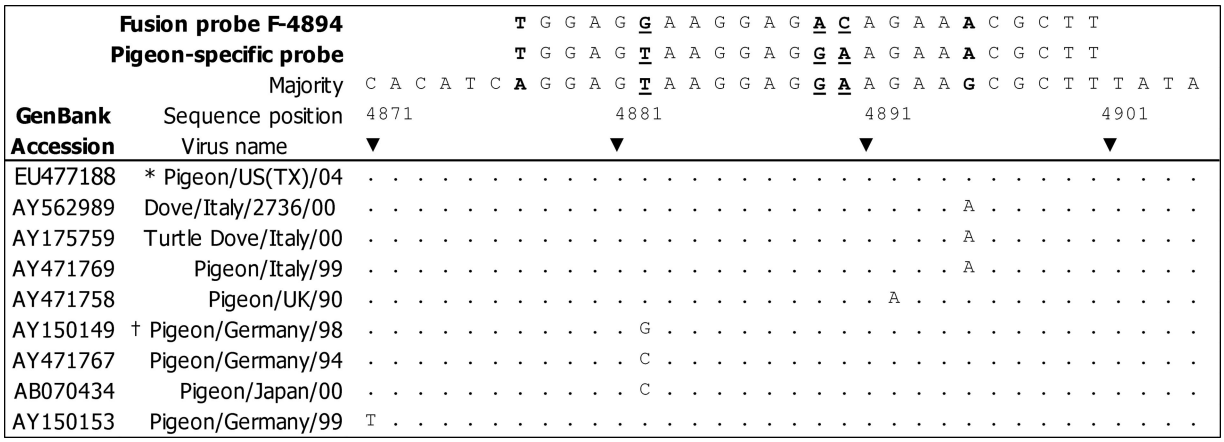

FIG. 1. Comparison of PPMV-1 sequences which encode the amino acid motif ^{112}RRKKRF^{117} at the fusion cleavage site ($n = 53$) to the fusion gene real-time RT-PCR assay probe and the pigeon-specific probe. Mismatches with the fusion probe sequences are in bold; changes made to the pigeon-specific probe are underlined. *, Sequence identical for viruses not shown ($n = 41$), i.e., those with accession no. EU477189 to -202, AB070419, AB070420, AB070422, AB070423, AB070426, AY175753, AY150129, AY150132, AY150133, AY150134, AY150135, AY150139, AY150140, AY150141, AY150143, AY445669, AY471757, AY471759, AY471760, AY471761, AY471763, AY471764, AY471765, AY471766, AY471768, and AY471770. †, Sequence identical for viruses not shown ($n = 3$), i.e., those with accession no. AY150150, AY150151, and AY471771.

virus circulating in the United States from 2000 to 2007. The present study involved the biological and phylogenetic characterization of PPMV-1 strains circulating in North American pigeons and doves, as well as the use of the USDA-validated M- and F-gene real-time RT-PCR assays; it also evaluated the use of an alternative F-gene probe for this viral subgroup.

MATERIALS AND METHODS

Isolates and sequence data. NDVs were obtained as part of West Nile virus surveillance programs in Rhode Island and the Houston metropolitan area from 2000 to 2007. Brain tissue was collected from dead birds and cultured in Vero cells as described previously (27, 37). Initial virus identification as NDV was made by a complement fixation (CF) test performed on fluids of cultures showing a viral cytopathic effect. NDV was isolated only from birds of the family Columbidae (Table 1).

Lyophilized Vero cell culture material was received at the Southeast Poultry Research Laboratory, reconstituted in 500 μl nuclease-free autoclaved water, and propagated in embryonated eggs. RNA was extracted from allantoic fluids with Trizol LS (Invitrogen, Carlsbad, CA) according to manufacturer instructions as previously described (21). All sequencing reactions were performed with fluorescent dideoxynucleotide terminators in an automated sequencer (ABI 3730XL automated sequencer; Applied Biosystems Inc., Foster City, CA). Nucleotide sequence editing and analyses were conducted with the LaserGene sequence analysis software package (LaserGene, version 5.07; DNAStar, Inc., Madison, WI). With the full-length genome positions from the NDV LaSota vaccine strain complete genome (accession no. AF077761), the homologous regions sequenced were a 374-bp partial F gene sequence (positions 4554 to 4917; $n = 10$) and the complete coding region of the F gene (positions 4544 to 6205; $n = 5$).

Hemagglutination (HA) and HA inhibition (HI) assays. The HA and HI assays were completed by microtiter methods. The HA assay of allantoic fluids harvested from inoculated embryonating eggs was used to identify NDV-positive embryos. Confirmation of NDV-positive fluids and antigenic characterization of virus isolates was conducted by HI with microtiter methods as previously described (22). Four HA units of viral test antigen was used in completing the HI assay with monoclonal antibodies (MAbs) and polyclonal antiserum.

MAbs. Five MAbs with different NDV specificities were used for differentiating selected isolates by the HI assay as previously described (22, 23). The MAbs, obtained from the USDA APHIS National Veterinary Services Laboratories (Ames, IA), included B79, 15C4, 10D11 (26), AVS (34), and 617/161 (13),

TABLE 2. MAb reactivity patterns of the PPMV-1 isolates used in this study[a]

Identification no.	GenBank accession no.	Isolate description	Reactivity with:				
			AVS	B79	15C4	10D11	161/167
447	EU477200	Mourning Dove/US/TX4048/2004	−	−	−	−	+
435	EU477194	Eurasian Collared Dove/US/TX3817/2004	−	+	−	−	+
436	EU477195	Eurasian Collared Dove/US/TX6295/2006	−	+	−	−	+
437	EU477196	Eurasian Collared Dove/US/TX6306/2007	−	+	−	−	+
438	EU477197	Eurasian Collared Dove/US/TX6338/2007	−	+	−	−	+
440	EU477188	Dove/US/TX-B2580/2004	−	+	−	−	+
446	EU477191	Eurasian Collared Dove/US/TX3988/2004	−	+	−	−	+
448	EU477201	Eurasian Collared Dove/US/TX4078/2004	−	+	−	−	+
449	EU477202	Pigeon/US/TX4142/2005	−	+	−	−	+
450	EU477192	Eurasian Collared Dove/US/TX4156/2005	−	+	−	−	+
434	EU477193	Eurasian Collared Dove/US/TX2334/2003	−	+	+	−	+
441	EU477189	Pigeon/US/RI166/2000	−	+	+	−	+
443	EU477198	Pigeon/US/TX3377/2004	−	+	+	−	+
444	EU477190	Pigeon/US/TX3503/2004	−	+	+	−	+

[a] $n = 14$.
[b] Polyclonal chicken antiserum served as the positive control and produced titers of ≥640 for each virus; the negative control titers were <2.

TABLE 3. C_t values from three real-time RT-PCR assays[a]

Identification no.	GenBank accession no.	Isolate description	Matrix	Fusion	Revised fusion	ICPI
434	EU477193	Eurasian Collared Dove/US/TX2334/2003	16.83	0	23.55	ND[b]
435	EU477194	Eurasian Collared Dove/US/TX3817/2004	18.03	0	24.66	ND
436	EU477195	Eurasian Collared Dove/US/TX6295/2006	16.71	0	23.55	1.13
437	EU477196	Eurasian Collared Dove/US/TX6306/2007	15.36	0	21.63	1.31
438	EU477197	Eurasian Collared Dove/US/TX6338/2007	17.33	0	24.59	1.26
440	EU477188	Dove/US/TX-B2580/2004	16.53	0	22.3	0.98
441	EU477189	Pigeon/US/RI166/2000	18.32	0	19.97	1.3
443	EU477198	Pigeon/US/TX3377/2004	16.22	0	21.45	1.29
444	EU477190	Pigeon/US/TX3503/2004	19.24	0	25.77	1.26
445	EU477199	Eurasian Collared Dove/US/TX3908/2004	18.6	0	27.26	ND
446	EU477191	Eurasian Collared Dove/US/TX3988/2004	17.79	0	24.44	1.15
447	EU477200	Mourning Dove/US/TX4048/2004	17.81	0	23.46	1.09
448	EU477201	Eurasian Collared Dove/US/TX4078/2004	17.07	0	24.33	ND
449	EU477202	Pigeon/US/TX4142/2005	17.66	0	27.54	1.14
450	EU477192	Eurasian Collared Dove/US/TX4156/2005	17.23	0	22.78	1.35

[a] The matrix gene assay, the fusion gene assay, and the revised fusion gene assay with a pigeon-specific probe ($n = 15$) were used. The associated ICPIs for selected PPMV-1 isolates ($n = 11$) are shown. C_t values of 1 to 35 are considered positive. Virulent isolates have ICPIs of >0.7 and phenylalanine (F) at position 117 (all isolates exhibited ^{112}RRKKRF^{117}).

[b] ND, not done.

and their reactivity has been described previously (21). A positive result was defined as antibody-inhibited HA, and a negative result was defined as no HI.

Pathogenicity assessment. The pathogenic potential of selected pigeon and dove isolates was evaluated by using standard assay methods to determine the intracerebral pathogenicity index (ICPI) in 1-day-old chicks (4).

Real-time RT-PCR. Alternate probes were designed for the F-gene assay from the consensus of an alignment (Fig. 1) which included the PPMV-1 strains reported here ($n = 15$), as well as other reference sequences encoding the amino acid motif ^{112}RRKKRF^{117} at the fusion cleavage site ($n = 38$). All pigeon viruses ($n = 15$) were tested by using the USDA-validated M-gene and F-gene assay protocols (40) and a modified F-gene assay with a degenerate probe and a PPMV-1-specific probe following the original M-gene assay protocol.

Phylogenetic analysis. Maximum-likelihood (ML) phylogenetic analysis with bootstrap values for 100 replicates was performed with Phyml under the general time-reversible model of nucleotide substitutions, ML estimates of base frequencies, the estimated transition/transversion ratio, and proportions of invariable sites with four categories of substitution rates (17). The full coding region of the F gene from pigeon isolates ($n = 5$) was compared to reference sequences representing known clades and genotypes ($n = 74$). The 374-bp region of the F gene, which has commonly been used for phylogenetic analysis of NDV (3), was sequenced to localize the U.S. pigeon viruses among other class II genotype VI reference sequences ($n = 47$).

Serologic comparison of two isolates. Immune sera to two of the NDV isolates (Pigeon/US/RI166/2000 and Eurasian Collared Dove/US/TX2334/2003) were prepared in adult mice at the University of Texas. The immunizing antigens were homogenates of brains of newborn mice inoculated intracerebrally with the respective viruses. CF tests were performed by the microtiter technique as previously described (11). Neutralization tests were performed by a plaque reduction neutralization method as previously described (33) in 24-well microplate cultures of Vero cells with a constant virus inoculum (~100 PFU) against twofold antiserum dilutions ranging from 1:10 to 1:1,280. The highest dilution of antiserum that reduced the virus by >90% was considered the antibody titer.

Nucleotide sequence accession numbers. The sequences of the 15 U.S. pigeon and dove isolates reported here have the following GenBank accession numbers: F gene, EU477188 to EU477192; 374-bp fragment, EU477193 to EU477202 (Table 1). The accession numbers of previously published sequences used in the analyses are in Table 1.

RESULTS

As part of a West Nile virus surveillance program in the Houston metropolitan area and in Rhode Island from 2000 to 2007, brain tissue from 5,608 dead birds representing 21 avian orders was cultured in Vero cells. Fifteen NDV isolates were recovered from 1,416 birds of the order Columbiformes, family Columbidae (Table 1). Isolates were serologically characterized with a panel of five different MAbs. The reactivity of PPMV-1 strains in the MAbs tested is typically as follows: AVS, negative; B79, positive; 15C4, negative; 10D11, negative; 617/161, positive. Nine of the isolates tested demonstrated the typical pattern of PPMV-1, with reaction to B79 and 617/161 (Table 2; $n = 14$). One isolate, Mourning Dove/US/TX4048/2004 (04US447MDV), exhibited a normal variation for PPMV-1, binding only to the 617/161 MAb. The remaining four isolates presented an atypical pattern, binding to 15C4, as well as B79 and 617/161.

All isolates were determined to be virulent according to the World Organization for Animal Health standard (10), which states that virulent viruses have an ICPI of ≥0.7 or encode multiple basic amino acids at the C terminus of the F2 protein and have phenylalanine at residue 117. Each virus encoded a virulence fusion cleavage site motif (^{112}RRKKRF^{117}), and the ICPIs of selected isolates ($n = 11$) ranged from 0.98 to 1.35 (Table 3).

Initial testing demonstrated that all of the isolates were positively identified by the USDA-validated real-time RT-PCR M-gene assay (cycle threshold [C_t] range, 15.36 to 19.24); however, none were detected with the F-gene assay after duplicate attempts (C_t, 0; Table 3). To determine whether mismatches at the probe site were responsible for the F-gene assay failure, a PPMV-1-specific probe was designed by evaluating a 24-nt alignment of PPMV-1 sequences (positions 4871 to 4894) which encode the ^{112}RRKKRF^{117} motif compared to the F-gene assay probe site (Fig. 1). This region was identical in the 15 PPMV-1 strains described here and 27 other viruses from Japan, South Africa, Spain, Austria, Germany, Denmark, France, Ireland, and the United Kingdom. Three mismatches that were present in the PPMV-1 sequences compared to the 24-nt F-gene probe sequence (position 6, G to T; position 13, A to G; position 14, C to A) were chosen to make a new probe that successfully detected all PPMV-1 isolates (Table 3; $n = 15$). Based on results from a previous study (20), an additional probe was designed with a single degenerate site at position 6

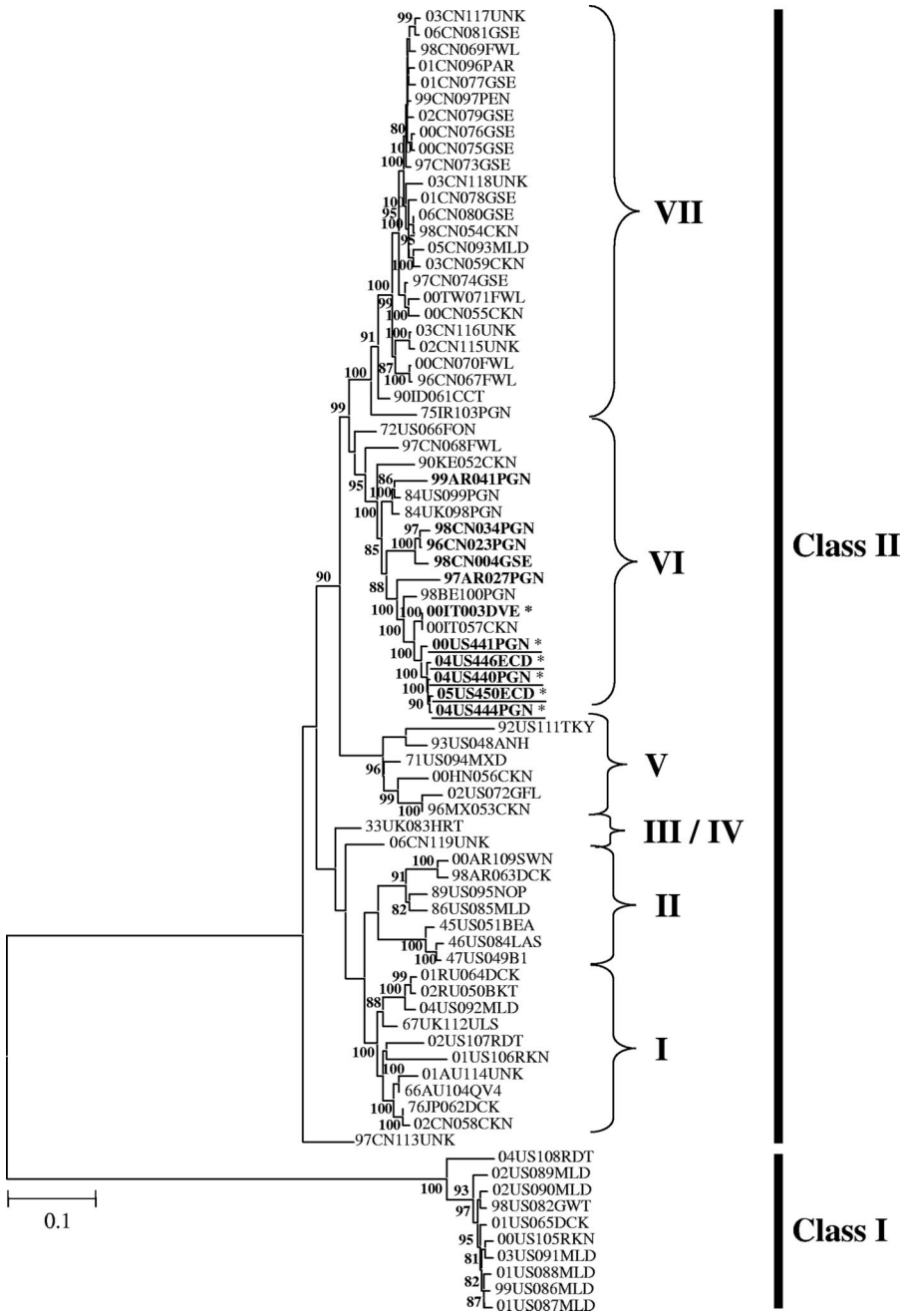

FIG. 2. Phylogenetic analysis of the full coding region of the fusion gene for PPMV-1 ($n = 5$; underlined) with reference sequences representing recognized genotypes in class II (I to VII, denoted at right; $n = 64$) and class I ($n = 10$). Asterisks represent isolates known to escape detection by the fusion gene assay. Names in bold also appear in the 374-bp fusion gene fragment phylogenetic analysis (see Fig. 3). The tree was constructed by Phyml ML with 100 bootstrap replicates. The scale indicates the branch length based on the number of nucleotide substitutions per site. Virus designations represent a 10-character name containing the two-digit year of collection, the two-letter ISO country code abbreviation, the three-digit unique virus identification number, and the three-letter species abbreviation (refer to Table 1).

(G to K = G/T); however, none of the isolates tested in this study were detected with the degenerate probe (data not shown).

To determine the distribution of these viruses in comparison to known clades and genotypes, sequencing and analysis of the full coding region of the F gene were performed (Fig. 2). The pigeon viruses clearly fall within the other genotype VI virus group. Among the PPMV-1 strains reported here, the 00US441PGN virus forms a separate branch likely because it is geographically separated (Rhode Island) from the other viruses, which were isolated in Texas. The most closely related isolates are a chicken and a dove isolate from Italy (00IT057CKN and 00IT003DVE) and a pigeon isolate from Belgium (98BE100PGN). The 00IT003DVE isolate (20) is included among isolates that were

TABLE 4. Results of plaque reduction neutralization assay with PPMV-1 isolates Pigeon/US/RI166/2000[a] and Eurasian Collared Dove/US/TX2334/2003[b]

Identification no.	GenBank accession no.	Virus	Antibody dilution[c]	
			441	434
441	EU477189	Pigeon/US/RI166/2000	1:160	1:20
434	EU477193	Eurasian Collared Dove/US/TX2334/2003	1:20	1:160

[a] Identification no. 441.
[b] Identification no. 434.
[c] Highest antibody dilution giving >90% plaque reduction.

not detected by the F-gene assay (Fig. 2; denoted by asterisks). Results of the plaque reduction neutralization assay comparing two PPMV-1 strains, Pigeon/US/RI166/2000 (00US441PGN) and Eurasian Collared Dove/US/TX2334/2003 (03US434ECD), demonstrate that these two isolates were distinct and support the results of a phylogenetic analysis (Table 4); however, the isolates were indistinguishable by the CF assay (data not shown).

Closer phylogenetic evaluation of genotype VI viruses with the 374-bp fragment suggests that the pigeon and dove viruses reported here belong to a subgroup, VIb/4bii (VIb refs; 4b refs), many of the members of which encode the ^{112}RRKKRF^{117} motif at the fusion cleavage site (Fig. 3). Fourteen of the pigeon and dove viruses described here form a distinct cluster (designated TX, Fig. 3). While these viruses likely represent a geographical separation, it is interesting that the dates of isolation span 5 years (2003 to 2007). Viruses collected in 16 countries all over the world from 1992 to 2007 (51/52) are found among the VIb/4bii subgroup, suggesting broad geographic dispersal of this relatively recent subgroup. As with the full coding sequence analysis, the pigeon isolate 00US441PGN (Rhode Island) clusters separately from the TX viruses and groups together with a recent pigeon isolate from Quebec, Canada (06CA047PGN).

DISCUSSION

Monitoring efforts in the Houston metropolitan area identified PPMV-1 in dead pigeons and doves from 2003 to 2007, suggesting that these viruses may be endemic and circulating in the United States. These findings are consistent with previous reports suggesting that, despite widespread vaccination efforts, the PPMV-1 identified during the mid 1980s panzootic appears to have become endemic in areas maintaining large populations of Columbiformes birds (2, 5, 31). The majority (67% [951/1,416]) of the bird samples were received during the warmer months of May to September (average temperatures, 75.8 to 78.9°F); however, 10 of the 15 NDV isolations were from samples obtained during the cooler months (October to April; average temperatures, 51.8 to 70.4°F). The reason for the lower rate of isolation during warmer months is unknown; high temperatures may affect the stability of the virus in the environment, resulting in lower rates of transmission, or may cause inactivation of the virus in bird carcasses during the summer months.

Previous efforts to characterize the pathogenicity of various PPMV-1 strains in poultry revealed variability in the results of pathogenicity assays performed with chickens, even though the isolates typically had the recognized virulence fusion protein cleavage site motif ^{112}G/RRQKRF^{117} (14, 22, 31). The isolates described in the present report encode a virulence fusion cleavage site motif ^{112}RRKKRF^{117} and exhibit ICPIs ranging from 0.98 to 1.35 (Table 3), and these results are in agreement with previous studies characterizing isolates which encode the ^{112}RRKKRF^{117} cleavage site (31, 35, 39).

A USDA-validated real-time RT-PCR assay (F-gene assay) directed at the fusion cleavage site of NDV differentiates virulent strains from those of low virulence (40). During the initial evaluation of the F-gene assay, one virulent PPMV-1 isolate, DoveIT, escaped detection and it was postulated that

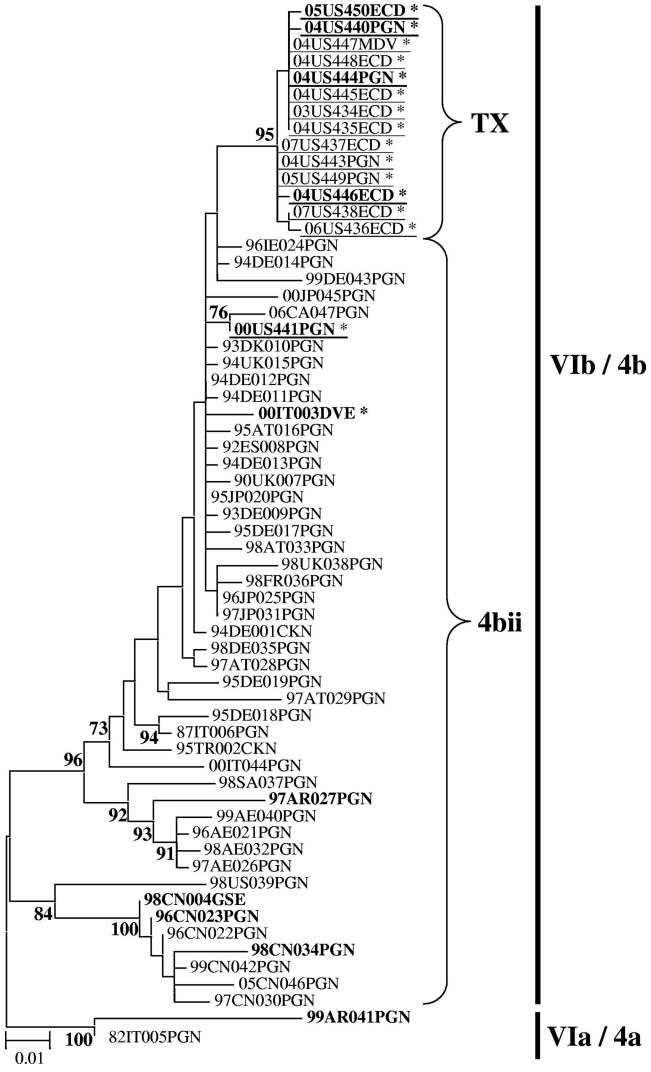

FIG. 3. Phylogenetic analysis of the 374-bp fusion gene fragment of PPMV-1 (n = 15; underlined) with reference sequences from class II genotype VI (n = 47). Asterisks represent isolates known to escape detection by the fusion gene assay. Names in bold also appear in the 374-bp fusion gene fragment phylogenetic analysis (Fig. 2). The tree was constructed by Phyml ML with 100 bootstrap replicates. The scale indicates the branch length based on the number of nucleotide substitutions per site. Virus designations represent a 10-character name containing the two-digit year of collection, the two-letter ISO country code abbreviation, the three-digit unique virus identification number, and the three-letter species abbreviation (refer to Table 1).

viruses encoding the ^{112}RRKKRF^{117} motif would escape detection because of the presence of three mismatches along the probe site sequence which prevented successful binding of the probe (20). This hypothesis held true for the PPMV-1 isolates reported here. The redesigned pigeon-specific probe successfully identified the PPMV-1 isolates as virulent, whereas the original F-gene probe failed. Further analysis suggested that a single nucleotide change (position 6 of the F-gene probe) may allow the probe to bind (20); however, the degenerate probe designed for the present study failed to bind. While these isolates were correctly identified by the M-gene assay, the accurate identification of virulent isolates is important to continued monitoring efforts. Therefore, inclusion of additional primers may need to be considered as part of the USDA surveillance plan.

The class II genotype VI group contains viruses from around the world collected as early as 1978 (19). This is a diverse group of viruses that have been phylogenetically characterized by many different authors into various sublineages (2, 16, 28–30, 38). Four isolates obtained from racing pigeons (*Columba livia*) fell outside the major grouping and encoded an unusual amino acid fusion cleavage site motif with lysine replacing glutamine at residue 114, producing the motif ^{112}RRKKRF^{117} (31). Viruses characterized by the ^{112}RRKKRF^{117} motif have been reported by several authors and include viruses from every major continent (1, 2, 31, 35, 38, 39). In each case, viruses encoding the ^{112}RRKKRF^{117} motif tend to cluster together separately from other genotype VI viruses but clearly remain within the genotype VI domain.

An outbreak described in ECD in Italy in 2000 and 2001 found 18/20 ECD isolates to cluster together regardless of geographic origin and postulated that these viruses represented a distinct sublineage circulating within a species (35); however, the present study found viruses with 100% identity along the 374-bp fragment of the fusion gene circulating in both pigeons and ECD clustered according to geographic origin. Our results show that viruses found within the VIb/4bii subgroup are temporally clustered from 1992 to 2007, which is in agreement with Aldous et al., who postulated that subgroup 4bii (1990s to 2000) was becoming the predominant sublineage over 4bi (representing older viruses from the 1980s to the 1990s) and may reflect selective pressure from vaccine usage (2).

PPMV-1 represents a significant ongoing threat to domestic and wild bird populations, and further understanding of the natural ecology and the effect of selective pressures on these viruses is needed. Additionally, PPMV-1 detected in U.S. urban pigeon and dove populations deserves continued investigation since previous outbreaks in the United Kingdom were thought to originate from feed contaminated with pigeon feces and introduced into naïve (unvaccinated) populations (8). The presence of these viruses in U.S. urban pigeons and doves, in addition to concerns about increased virulence of PPVM-1 upon replication in poultry, emphasizes the importance of control methods such as vaccination and monitoring in preventing PPMV-1 outbreaks in domestic poultry populations.

ACKNOWLEDGMENTS

We acknowledge Dawn Williams-Coplin and Tim Olivier for excellent technical assistance and the South Atlantic Area Sequencing Facility for nucleotide sequencing.

This work was funded by USDA CRIS project 6612-32000-049-00D and NIH contracts NO1-AI-25489 and NO1-AI30027 to R.B.T.

Mention of trade names or commercial products in this report is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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